Eur päisches Patentamt	European Pat nt Office	Office eur péen des brevets 08/716,169
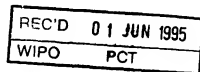
Bescheinigung   Certificate   Attestation

Die angehefteten Unterla- gen stimmen mit der ursprünglich eingereichten Fassung der auf dem näch- sten Blatt bezeichneten europäischen Patentanmel- dung überein.	The attached documents are exact copies of the European patent application described on the following page, as originally filed.	Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr.   Patent application No.   Demande de brevet n°

94200738. 6

PRIORITY DOCUMENT

Der Präsident des Europäischen Patentamts;
im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
P.O

D. RADFORD

Den Haag, den
The Hague,   23/03/95
La Haye, le

Blatt 2 der B scheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no :   94200738.6
Demande n°:

Anmeldetag:
Date of filing: 22/03/94
Date de dépôt

Anmelder:
Applicant(s):
Demandeur(s):

RIJKSUNIVERSITEIT TE UTRECHT

NL-3584 CS Utrecht

NETHERLANDS

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:

Pharmaceutical compostion for the treatment and prevention of inflammatory diseases and active components of such compositions

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no Numéro de dépôt:

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International Patent classification:
Classification internationale des brevets:

C12N15/00

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Contracting states designated at date of filing: AT/BE/CH/DE/DK/ES/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du dépôt:

Bemerkungen:
Remarks:
Remarques:

SEQ ID No. 1

```
Met Ala Lys Thr Ile Ala Tyr Asp Glu Glu Ala Arg Arg Gly Leu
              5                  10                      15

Glu Arg Gly Leu Asn Ala Leu Ala Asp Ala Val Lys Val Thr Leu
              20                 25                      30

Gly Pro Lys Gly Arg Asn Val Val Leu Glu Lys Lys Trp Gly Ala
              35                 40                      45

Pro Thr Ile Thr Asn Asp Gly Val Ser Ile Ala Lys Glu Ile Glu
              50                 55                      60

Leu Glu Asp Pro Tyr Glu Lys Ile Gly Ala Glu Leu Val Lys Glu
              65                 70                      75

Val Ala Lys Lys Thr Asp Asp Val Ala Gly Asp Gly Thr Thr Thr
              80                 85                      90

Ala Thr Val Leu Ala Gln Ala Leu Val Arg Glu Gly Leu Arg Asn
              95                 100                     105

Val Ala Ala Gly Ala Asn Pro Leu Gly Leu Lys Arg Gly Ile Glu
              110                115                     120

Lys Ala Val Glu Lys Val Thr Glu Thr Leu Leu Lys Gly Ala Lys
              125                130                     135

Glu Val Glu Thr Lys Glu Gln Ile Ala Ala Thr Ala Ala Ile Ser
              140                145                     150

Ala Gly Asp Gln Ser Ile Gly Asp Leu Ile Ala Glu Ala Met Asp
              155                160                     165

Lys Val Gly Asn Glu Gly Val Ile Thr Val Glu Glu Ser Asn Thr
              170                175                     180

Phe Gly Leu Gln Leu Glu Leu Thr Glu Gly Met Arg Phe Asp Lys
              185                190                     195

Gly Tyr Ile Ser Gly Tyr Phe Val Thr Asp Pro Glu Arg Gln Glu
              200                205                     210

Ala Val Leu Glu Asp Pro Tyr Ile Leu Leu Val Ser Ser Lys Val
              215                220                     225

Ser Thr Val Lys Asp Leu Leu Pro Leu Leu Glu Lys Val Ile Gly
              230                235                     240

Ala Gly Lys Pro Leu Leu Ile Ile Ala Glu Asp Val Glu Gly Glu
              245                250                     255

Ala Leu Ser Thr Leu Val Val Asn Lys Ile Arg Gly Thr Phe Lys
              260                265                     270

Ser Val Ala Val Lys Ala Pro Gly Phe Gly Asp Arg Arg Lys Ala
              275                280                     285
```

SEQ ID No. 1

Met Ala Lys Thr Ile Ala Tyr Asp Glu Glu Ala Arg Arg Gly Leu
                5   10   15

Glu Arg Gly Leu Asn Ala Leu Ala Asp Ala Val Lys Val Thr Leu
              20   25   30

Gly Pro Gly Lys Arg Asn Val Val Leu Glu Lys Lys Trp Gly Ala
              35   40   45

Pro Thr Ile Thr Asn Asp Gly Val Ser Ile Ala Lys Glu Ile Glu
              50   55   60

Leu Glu Asp Pro Tyr Glu Lys Ile Gly Ala Glu Leu Val Lys Glu
              65   70   75

Val Ala Lys Lys Thr Asp Asp Val Ala Gly Asp Gly Thr Thr Thr
              80   85   90

Ala Thr Val Leu Ala Gln Ala Leu Val Arg Glu Gly Leu Arg Asn
              95   100   105

Val Ala Ala Gly Ala Asn Pro Leu Gly Leu Lys Arg Gly Ile Glu
             110   115   120

Lys Ala Val Glu Lys Val Thr Glu Thr Leu Leu Lys Gly Ala Lys
             125   130   135

Glu Val Glu Thr Lys Glu Gln Ile Ala Ala Thr Ala Ala Ile Ser
             140   145   150

Ala Gly Asp Gln Ser Ile Gly Asp Leu Ile Ala Glu Ala Met Asp
             155   160   165

Lys Val Gly Asn Glu Gly Val Ile Thr Val Glu Glu Ser Asn Thr
             170   175   180

Phe Gly Leu Gln Leu Glu Leu Thr Glu Gly Met Arg Phe Asp Lys
             185   190   195

Gly Tyr Ile Ser Gly Tyr Phe Val Thr Asp Pro Glu Arg Gln Glu
             200   205   210

Ala Val Leu Glu Asp Pro Tyr Ile Leu Leu Val Ser Ser Lys Val
             215   220   225

Ser Thr Val Lys Asp Leu Leu Pro Leu Leu Glu Lys Val Ile Gly
             230   235   240

Ala Gly Lys Pro Leu Leu Ile Ile Ala Glu Asp Val Glu Gly Glu
             245   250   255

Ala Leu Ser Thr Leu Val Val Asn Lys Ile Arg Gly Thr Phe Lys
             260   265   270

Ser Val Ala Val Lys Ala Pro Gly Phe Gly Asp Arg Arg Lys Ala
             275   280   285

```
Met Leu Gln Asp Met Ala Ile Leu Thr Gly Gly Gln Val Ile Ser
            290                     295                     300

Glu Glu Val Gly Leu Thr Leu Glu Asn Ala Asp Leu Ser Leu Leu
            305                     310                     315

Gly Lys Ala Arg Lys Val Val Val Thr Lys Asp Glu Thr Thr Ile
            320                     325                     330

Val Glu Gly Ala Gly Asp Thr Asp Ala Ile Ala Gly Arg Val Ala
            335                     340                     345

Gln Ile Arg Gln Glu Ile Glu Asn Ser Asp Ser Asp Tyr Asp Arg
            350                     355                     360

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala
            365                     370                     375

Val Ile Lys Ala Gly Ala Ala Thr Glu Val Glu Leu Lys Glu Arg
            380                     385                     390

Lys His Arg Ile Glu Asp Ala Val Arg Asn Ala Lys Ala Ala Val
            395                     400                     405

Glu Glu Gly Ile Val Ala Gly Gly Gly Val Thr Leu Leu Gln Ala
            410                     415                     420

Ala Pro Thr Leu Asp Glu Leu Lys Leu Glu Gly Asp Glu Ala Thr
            425                     430                     435

Gly Ala Asn Ile Val Lys Val Ala Leu Glu Ala Pro Leu Lys Gln
            440                     445                     450

Ile Ala Phe Asn Ser Gly Leu Glu Pro Gly Val Val Ala Glu Lys
            455                     460                     465

Val Arg Asn Leu Pro Ala Gly His Gly Leu Asn Ala Gln Thr Gly
            470                     475                     480

Val Tyr Glu Asp Leu Leu Ala Ala Gly Val Ala Asp Pro Val Lys
            485                     490                     495

Val Thr Arg Ser Ala Leu Gln Asn Ala Ala Ser Ile Ala Gly Leu
            500                     505                     510

Phe Leu Thr Thr Glu Ala Val Val Ala Asp Lys Pro Glu Lys Glu
            515                     520                     525

Lys Ala Ser Val Pro Gly Gly Gly Asp Met Gly Gly Met Asp Phe
            530                     535                     540
```

SEQ. ID No. 2

The alignment was done on 4 Protein sequences.
Character to show that a position in the alignment is perfectly conserved:
'*'
Character to show that a position is well conserved: '.'

Alignment

```
  0$HUMAN    MLRLPTVFRQMRPVSRVLAPHLTRAYAKDVKFGADARALMLQGVDLLADA      50
   0$RAT     ------------------A--------KDVKFGADARALMLQGVDLLADA      24
  60$MOUSE   ------------------APHLTRAYAKDVKFGADARALMLQGVDLLADA      32
MBAA         M------------------------AKTIAYDEEARRGLERGLNALADA      25
                                     *.. ....**  .*.. ****

P60$HUMAN    VAVTMGPKGRTVIIEQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQD     100
P60$RAT      VAVTMGPKGRTVIIEQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQD      74
P60$MOUSE    VAVTMGPKGRTVIIEQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQD      82
MBAA         VKVTLGPKGRNVVLEKKWGAPTITNDGVSIAKEIELEDPYEKIGAELVKE      75
             * **.*****.*..*..**.*..*.***..**.*.*.* *..***.**..

P60$HUMAN    VANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEIRRGVMLAVDA     150
P60$RAT      VANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEIRRGVMLAVDA     124
P60$MOUSE    VANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEIRRGVMLAVDA     132
MBAA         VAKKTDDVAGDGTTTATVLAQALVREGLRNVAAGANPLGLKRGIEKAVEK     125
             **..*.. ************.....**. ... ****...**.  **.

P60$HUMAN    VIAELKKQSKPVTTPEEIAQVATISANGDKEIGNIISDAMKKVGRKGVIT     200
P60$RAT      VIAELKKQSKPVTTPEEIAQVATISANGDKDIGNIISDAMKKVGRKGVIT     174
P60$MOUSE    VIAELKKQSKPVTTPEEIAQVATISANGDKDIGNIISDAMKKVGRKGVIT     182
MBAA         VTETLLKGAKEVETKEQIAATAAISA-GDQSIGDLIAEAMDKVGNEGVIT     174
             *...*.  .* *.* *.**.*.*** **..**..*..**.***..****

P60$HUMAN    VKDGKTLNDELEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKK     250
P60$RAT      VKDGKTLNDELEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKK     224
P60$MOUSE    VKDGKTLNDELEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKK     232
MBAA         VEESNTFGLQLELTEGMRFDKGYISGYFVTDPERQEAVLEDPYILLVSSK     224
             *....*.. .**..***.**.**** **..... *. ..*.*.** ..*
```

```
P60$HUMAN   ISSIQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAV   300
P60$RAT     ISSVQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAV   274
P60$MOUSE   FSSVQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAV   282
MBAA        VSTVKDLLPLLEKVIGAGKPLLIIAEDVEGEALSTLVVNKIRGTFKSVAV   274
            *......* **  .  *** .*****.********.*.... ... ***

P60$HUMAN   KAPGFGDNRKNQLKDMAIATGGAVFGEEGLTLNLEDVQPHDLGKVGEVIV   350
P60$RAT     KAPGFGDNRKNQLKDMAIATGGAVFGEEGLNLNLEDVQAHDLGKVGEVIV   324
P60$MOUSE   KAPGFGDNRKNQLKDMAIATGGAVFGEEGLNLNLEDVQAHDLGKVGEVIV   332
MBAA        KAPGFGDRRKAMLQDMAILTGGQVISEE-VGLTLENADLSLLGKARKVVV   323
            *******.**. *.**** ***.*.** ..*.**...  ***..*.*

P60$HUMAN   TKDDAMLLKGKGDKAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSDG   400
P60$RAT     TKDDAMLLKGKGDKAHIEKRIQEITEQLDITTSEYEKEKLNERLAKLSDG   374
P60$MOUSE   TKDDAMLLKGKGDKAHIEKRIQEITEQLDITTSEYEKEKLNERLAKLSDG   382
MBAA        TKDETTIVEGAGDTDAIAGRVAQIRQEIENSDSDYDREKLQERLAKLAGG   373
            ***..**.* **.. *. *...* .... ...*.*..***.******..*

   0$HUMAN   VAVLKVGGTSDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALLRCIPA   450
   0$RAT     VAVLKVGGTSDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALLRCIPA   424
   0$MOUSE   VAVLKVGGTSDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALLRCIPA   432
MBAA        VAVIKAGAATEVELKERKHRIEDAVRNAKAAVEEGIVAGGGVTLLQAAPT   423
            ***..*.*....**..*.*.*..**.......******* *** .**. *.

P60$HUMAN   LDSLTPANEDQKIGIEIIKRTLKIPAMTIAKNAGVEGSLIVEKIMQSSSE   500
P60$RAT     LDSLKPANEDQKIGIEIIKRALKIPAMTIAKNAGVEGSLIVEKILQSSSE   474
P60$MOUSE   LDSLKPANEDQKIGIEIIKRALKIPAMTIAKNAGVEGSLIVEKILQSSSE   482
MBAA        LDELK-LEGDEATGANIVKVALEAPLKQIAFNSGLEPGVVAEKVRNLPAG   472
            **.*. ..*. .* .*.* .*. *. ** .**.... **.. . ....

P60$HUMAN   VGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVTEIP   550
P60$RAT     VGYDAMLGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEAVVTEIP   524
P60$MOUSE   VGYDAMLGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEAVVTEIP   532
MBAA        HGLNAQTGVYEDLLAAGVADPVKVTRSALQNAASIAGLFLTTEAVVADKP   522
            *.* * .... *. **.**.**.**..*.*. *.*.**.. *

 P60$HUMAN   KEEKDPGMGAMGGMGGGMGGGMF   573
    0$RAT    KEEKDPGMGAMGGMGGGMGGGMF   547
    .0$MOUSE  KEEKDPGMGAMGGMGGGMGGGMF   555
 (MBAA       EKEKASVPG-----GGDMGGGMDF  540
             ..**.. *    .**.*** *
```

Consensus length: 573
Identity : 254 (44.3%)
Similarity: 211 (36.8%)

SEQ ID No. 3

TRANSLATION OF NUCLEIC ACID SEQUENCE OF THE MYCOB. BOVIS BCG HSP65 GENE

```
      580               590            600            610            620
       |                 |              |              |              |
 ATG GCC AAG ACA ATT GCG TAC GAC GAA GAG GCC CGT CGC GGC CTC GAG CGG GGC
 Met Ala Lys Thr Ile Ala Tyr Asp Glu Glu Ala Arg Arg Gly Leu Glu Arg Gly  18

630             640            650            660            670            680
 |               |              |              |              |              |
 TTG AAC GCC CTC GCC GAT GCG GTA AAG GTG ACA TTG GGC CCC AAG GGC CGC AAC
 Leu Asn Ala Leu Ala Asp Ala Val Lys Val Thr Leu Gly Pro Lys Gly Arg Asn  36

      690               700            710            720            730
       |                 |              |              |              |
 GTC GTC CTG GAA AAG AAG TGG GGT GCC CCC ACG ATC ACC AAC GAT GGT GTG TCC
 Val Val Leu Glu Lys Lys Trp Gly Ala Pro Thr Ile Thr Asn Asp Gly Val Ser  54

   740             750            760            770            780            790
    |               |              |              |              |              |
 ATC GCC AAG GAG ATC GAG CTG GAG GAG CTG GAG GAT CCG TAC GAG GCC GAG CTG
 Ile Ala Lys Glu Ile Glu Leu Glu Glu Leu Glu Asp Pro Tyr Glu Ala Glu Leu  72

            800            810            820            830            840
             |              |              |              |              |
 GTC AAA GAG GTA GCC AAG AAG ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG ACG
 Val Lys Glu Val Ala Lys Lys Thr Asp Asp Val Ala Gly Asp Gly Thr Thr Thr  90

      850               860            870            880            890
       |                 |              |              |              |
 GCC ACC GTG CTG GCC CAG GCG TTG GTT CGC CAG GGC CTG CGC AAC GTC GCG GCC
 Ala Thr Val Leu Ala Gln Ala Leu Val Arg Gln Gly Leu Arg Asn Val Ala Ala 108

900             910            920            930            940            950
 |               |              |              |              |              |
 GGC GCC AAC CCG CTC GGT CTC AAA CGC GGC ATC GAA AAG GCC GTG GAG AAG GTC
 Gly Ala Asn Pro Leu Gly Leu Lys Arg Gly Ile Glu Lys Ala Val Glu Lys Val 126

         960              970            980            990           1000
          |                |              |              |              |
 ACC GAG ACC CTG CTC AAG GGC GCC AAG GAG GTC GAG ACC AAG GAG CAG ATT GCG
 Thr Glu Thr Leu Leu Lys Gly Ala Lys Glu Val Glu Thr Lys Glu Gln Ile Ala 144

   1010            1020           1030           1040           1050           1060
    |               |              |              |              |              |
 GCC ACC GCA GCG ATT TCG GCG GGT GAC CAG TCC ATC GGT GAC CTG ATC GCC GAG
 Ala Thr Ala Ala Ile Ser Ala Gly Asp Gln Ser Ile Gly Asp Leu Ile Ala Glu 162

         1070            1080           1090           1100           1110
          |                |              |              |              |
 GCG ATG GAC AAG GTG GGC AAC GAG GGC GTC ATC ACC GTC GAG GAG TCC AAC ACC
 Ala Met Asp Lys Val Gly Asn Glu Gly Val Ile Thr Val Glu Glu Ser Asn Thr 180
```

SEQ ID No. 3 (continued)

```
        1120            1130            1140            1150            1160
         |               |               |               |               |
TTT GGG CTG CAG CTC GAG CTC ACC GAG GGT ATG CGG TTC GAC AAG GGC TAC ATC
Phe Gly Leu Gln Leu Glu Leu Thr Glu Gly Met Arg Phe Asp Lys Gly Tyr Ile 198

1170        1180            1190            1200            1210            1220
  |           |               |               |               |               |
TCG GGG TAC TTC GTG ACC GAC CCG GAC CGT CAG GAG GCG GTC CTG GAG GAC CCC
Ser Gly Tyr Phe Val Thr Asp Pro Glu Arg Gln Glu Ala Val Leu Glu Asp Pro 216

        1230            1240            1250            1260            1270
         |               |               |               |               |
TAC ATC CTG CTG GTC AGC TCC AAG GTG TCC ACT GTC AAG GAT CTG CTG CCG CTG
Tyr Ile Leu Leu Val Ser Ser Lys Val Ser Thr Val Lys Asp Leu Leu Pro Leu 234

    1280            1290            1300            1310            1320            1330
     |               |               |               |               |               |
CTC GAG AAG GTC ATC GGA GCC GGT AAG CCG CTG CTG ATC ATC GCC GAG GAC GTC
Leu Glu Lys Val Ile Gly Ala Gly Lys Pro Leu Leu Ile Ile Ala Glu Asp Val 252

            1340            1350            1360            1370            1380
             |               |               |               |               |
GAG GGC GAG GCG CTG TCC ACC CTG GTC GTC AAC AAG ATC CGC GGC ACC TTC AAG
Glu Gly Glu Ala Leu Ser Thr Leu Val Val Asn Lys Ile Arg Gly Thr Phe Lys 270

    1390            1400            1410            1420            1430
     |               |               |               |               |
TCG GTG GCG GTC AAG GCT CCC GGC TTC GGC GAC CGC CGC AAG GCG ATG CTG CAG
Ser Val Ala Val Lys Ala Pro Gly Phe Gly Asp Arg Arg Lys Ala Met Leu Gln 288

1440        1450            1460            1470            1480            1490
  |           |               |               |               |               |
GAT ATG GCC ATT CTC ACC GGT GGT CAG GTG ATC AGC GAA GAG GTC GGC CTG ACG
Asp Met Ala Ile Leu Thr Gly Gly Gln Val Ile Ser Glu Glu Val Gly Leu Thr 306

        1500            1510            1520            1530            1540
         |               |               |               |               |
CTG GAG AAC GCC GAC CTG TCG CTG CTA GGC AAG GCC CGC AAG GTC GTG GTC ACC
Leu Glu Asn Ala Asp Leu Ser Leu Leu Gly Lys Ala Arg Lys Val Val Val Thr 324

    1550            1560            1570            1580            1590            1600
     |               |               |               |               |               |
AAG GAC GAG ACC ACC ATC GTC GAG GGC GCC GGT GAC ACC GAC GCC ATC GCC GGA
Lys Asp Glu Thr Thr Ile Val Glu Gly Ala Gly Asp Thr Asp Ala Ile Ala Gly 342

            1610            1620            1630            1640            1650
             |               |               |               |               |
CGA GTG GCC CAG ATC CGC CAG GAG ATC GAG AAC AGC GAC TCC GAC TAC GAC CGT
Arg Val Ala Gln Ile Arg Gln Glu Ile Glu Asn Ser Asp Ser Asp Tyr Asp Arg 360

    1660            1670            1680            1690            1700
     |               |               |               |               |
GAG AAG CTG CAG GAG CGG CTG GCC AAG CTG GCC GGT GGT GTC GCG GTG ATC AAG
Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val Ile Lys 378
```

SEQ ID No. 3 (continued)

```
1710        1720          1730         1740        1750          1760
 |           |             |            |           |             |
GCC GGT GCC GCC ACC GAG GTC GAA CTC AAG GAG CGC AAG CAC CGC ATC GAG GAT
Ala Gly Ala Ala Thr Glu Val Glu Leu Lys Glu Arg Lys His Arg Ile Glu Asp 396

       1770          1780          1790        1800         1810
        |             |             |           |            |
GCG GTT CGC AAT GCC AAG GCC GCC GTC GAG GAG GGC ATC GTC GCC GGT GGG GGT
Ala Val Arg Asn Ala Lys Ala Ala Val Glu Glu Gly Ile Val Ala Gly Gly Gly 414

  1820          1830          1840         1850         1860         1870
   |             |             |            |            |            |
GTG ACG CTG TTG CAA GCG GCC CCG ACC CTG GAC GAG CTG AAG CTC GAA GGC GAC
Val Thr Leu Leu Gln Ala Ala Pro Thr Leu Asp Glu Leu Lys Leu Glu Gly Asp 432

          1880          1890          1900        1910         1920
           |             |             |           |            |
GAG GCG ACC GGC GCC AAC ATC GTG AAG GTG GCG CTG GAG GCC CCG CTG AAG CAG
Glu Ala Thr Gly Ala Asn Ile Val Lys Val Ala Leu Glu Ala Pro Leu Lys Gln 450

   1930          1940          1950        1960         1970
    |             |             |           |            |
ATC GCC TTC AAC TCC GGG CTG GAG CCG GGC GTG GTG GCC GAG AAG GTG CGC AAC
Ile Ala Phe Asn Ser Gly Leu Glu Pro Gly Val Val Ala Glu Lys Val Arg Asn 468

1980        1990          2000        2010         2020          2030
 |           |             |           |            |             |
CTG CCG GCT GGC CAC GGA CTG AAC GCT CAG ACC GGT GTC TAC GAG GAT CTG CTC
Leu Pro Ala Gly His Gly Leu Asn Ala Gln Thr Gly Val Tyr Glu Asp Leu Leu 486

       2040          2050          2060        2070         2080
        |             |             |           |            |
GCT GCC GGC GTT GCT GAC CCG GTC AAG GTG ACC CGT TCG GCG CTG CAG AAT GCG
Ala Ala Gly Val Ala Asp Pro Val Lys Val Thr Arg Ser Ala Leu Gln Asn Ala 504

  2090          2100          2110        2120         2130         2140
   |             |             |           |            |            |
GCG TCC ATC GCG GGG CTG TTC CTG ACC ACC GAG GCC GTC GTT GCC GAC AAG CCG
Ala Ser Ile Ala Gly Leu Phe Leu Thr Thr Glu Ala Val Val Ala Asp Lys Pro 522

          2150          2160          2170        2180         2190
           |             |             |           |            |
GAA AAG GAG AAG GCT TCC GTT CCC GGT GGC GGC GAC ATG GGT GGC ATG GAT TTC
Glu Lys Glu Lys Ala Ser Val Pro Gly Gly Gly Asp Met Gly Gly Met Asp Phe 540

   2200
    |
TGA CCC
 -  Pro
```

Pharmaceutical composition for the treatment and prevention of inflammatory diseases and active components of such compositions

The invention pertains to polypeptides containing a part of the aminoacid sequence of the heat shock protein hsp65 of *Mycobacterium*
5 *tuberculosis* which polypeptides are capable of immunizing against arthritis and other inflammatory diseases and/or curing such diseases, as well as to nucleotide sequences encoding such polypeptides, cells and microorganisms expressing such polypeptides and pharmaceutical and diagnostic compositions containing such polypeptides.
10   It was found that immunisation of rats with a peptide corresponding to sequence 256-265 of SEQ ID No.1 induced strong protection against induction, seven days later, of adjuvant arthritis (AA). This finding was confirmed when using peptide 256-270. Immunisation with a peptide corresponding to sequence 91-100 of SEQ ID No.1 induced moderate protection,
15 whereas immunisation with peptides corresponding to the other epitopes produces little or no protection against adjuvant arthritis.
       The T cell line H.52, originally generated from hsp65 immunised rats and specific for epitope 256-265 also showed a protective effect on AA development when injected i.v. at the time of administration of *Myco*
20 *bacterium tuberculosis*.
       It is concluded that protective epitopes in hsp65 are located at positions where at least 5 aminoacids are in the same relative position as the same aminoacids in a T cell epitope of hsp65 that contains at least 4 consecutive aminoacids which are identical with the corresponding mammalian
25 hsp60 aminoacids. Mammalian hsp includes human, rat and mouse hsp. The human, rat, mouse and mycobacterial hsp60/hsp65 aminoacid sequences are depicted in one letter code in SEQ ID No. 2. The aminoacids which are identical are also shown in SEQ ID No. 2.
       The polypeptides are especially those having 5 aminoacids which are
30 in the same relative position as the same aminoacids in one of the sequences 81-100 and 241-270 of SEQ ID No. 1, more particularly having at least 5 aminoacids which are in the same relative position as the same aminoacids in one of the sequences 84-95 and 256-265 of SEQ ID No. 1. With the preference, the polypeptides comprise at least 7 aminoacids with the same
35 relative positions as those in the hsp65 T cell epitopes. Those epitopes are especially those which have at least 4 consecutive aminoacids which are identical with the corresponding mammalian hsp60 aminoacids. Examples of

suitable polypeptide comprise the sequences [Ala Thr Val Leu Ala], [Ala Leu Ser Thr Leu] and [Leu Ser Thr Leu Val]. In particular, the polypeptide comprises 5-30 aminoacids of the amino acid sequence of hsp65; these hsp65 aminoacids may be coupled to other sequences, such as spacer sequences or
5   fused peptide sequences.

The polypeptides are suitable for protecting against inflammatory diseases, including autoimmune diseases, diabetes, arthritide diseases, atherosclerosis, multiple sclerosis, and myasthenia gravis.

The invention also concerns polypeptide analogues which exhibit the
10   immunological properties of the polypeptides described above, but which contain one or chemical modifications. Such polypeptide analogues, also referred to as peptidomimetics, can e.g. consist of units corresponding to the aminoacid residues of the polypeptides described above, wherein essentially the same side groups are present, but wherein the backbone
15   contains modifications such as substitution of an amide group (CO-NH) by another group such as CH=CH, CO-O, CO-CH$_2$ or CH$_2$-CH$_2$. Other modifications, such as substitutions of an aminoacid by a similar natural, or non-natural aminoacid are also envisaged.

The invention furthermore relates to pharmaceutical compositions
20   suitable for protection against or treatment of an inflammatory disease, including autoimmune diseases, diabetes, arthritide diseases, multiple sclerosis and myasthenia gravis, containing a polypeptide as described above or a nucleotide sequence, an expression system, a cell (eukaryotic) or microorganism corresponding to and/or encoding such polypeptide. The
25   composition may be in the form of a vaccine; it can then also contain a conventional adjuvant, such as Freund's complete or incomplete adjuvant or other adjuvant, and/or carrier materials and other additives.

The composition may also be in the form of a medicine suitable for curing developing or developed inflammatory diseases; it contains
30   conventional additives and excipients. As a treatment composition, it may also contain an antibody against the polypeptides described above.

The invention also relates to diagnostics means and methods based on the polypeptides described above, or the corresponding antibodies or nucleotide sequences (probes).
35   SEQ. ID No 3. contains the nucleotide sequence and aminoacid sequence of hsp65. Sequences 84-95 and 256-270 correspond to protective polypeptides.

Abbreviations used in this description:

AA, adjuvant arthritis

DDA: dimethyl dioctadecyl ammonium bromide;

hsp60: mammalian 60 kDa heat shock protein

hsp65: mycobacterial 65 kDa heat shock protein

IFA: incomplete Freund's adjuvant

JCA: juvenile chronic arthritis.

Mt: heat-killed *Mycobacterium tuberculosis*

PPD: purified protein derivative of *M.tuberculosis*

PLNC: primed lymph node cells

RA, rheumatoid arthritis

Background

Adjuvant arthritis (AA), an extensively studied model of human rheumatoid arthritis (RA) or reactive arthritis. As the pathogenic mechanisms underlying RA remain unclear, extensive use is made of experimental rodent arthritis models. Lewis rats are susceptible to arthritis following administration of various arthritogenic preparations including heat-killed *M. tuberculosis* (Mt) suspended in IFA (adjuvant arthritis or, AA) (1), streptococcal cell walls (SCW-arthritis), collagen type II and the lipoidal amine CP20961. The cellular basis for AA induction was demonstrated by the passive transfer of the disease to naive rats using splenocytes from arthritic rats (2). Induction of disease in irradiated naive rats by administration of the Mt-reactive T cell clone A2b was reported (3,4). The Ag-specificity of A2b was identified as residues 180-188 of the mycobacterial 65 kDa heat shock protein (hsp65) (5). Attempts to induce AA by immunisation with hsp65 alone proved unsuccessful. Instead, this approach conferred resistance to subsequent attempts to induce AA with whole Mt (5,6). This protective effect is believed to be mediated by T cells specific for hsp65 (7). Preimmunisation with mycobacterial hsp65 has subsequently been reported to confer protection against other forms of experimental arthritis induced with streptococcal cell walls (8), collagen type II (6,9), or synthetic adjuvants such as CP20961 (6) and pristane (10).

Mycobacterial hsp65 belongs to the hsp60 family of heat shock proteins which is highly conserved throughout evolution, and shares 48% aminoacid identity with the mammalian homologue, P1 or hsp60 (11). Expression of mammalian hsp60 is known to be upregulated as a physiological

response to various stressful stimuli, and has been shown to be elevated in inflamed synovia of patients with RA (12),or juvenile chronic arthritis (JCA, ref.13). There have been numerous reports of cross-reactive immuno-recognition of mycobacterial hsp65 and endogenous self hsp60 at the T cell

5   level (14-17). This has led to the suggestion that T cells specific for self hsp60 might in some way play a role in the regulation of the inflamma-tory response (16). Further, this might account for the protective effect of preimmunisation with hsp65 via activation of T cells capable of recogn-ising self hsp60 and leading to improved regulation of future inflammatory

10   episodes induced by administration of arthritogenic substances. Alter-natively, immunisation with hsp65 might induce T cell responses to myco-bacterial-unique epitopes which are not induced by the Mt administration protocol used to induce AA and these responses might mediate the protective mechanism.

15   Materials and Methods
Animals: Male inbred Lewis rats (RT1[1] MHC haplotype) were obtained from the University of Limburg, Maastricht, The Netherlands. Rats were five to eight weeks old at the start of each experiment.

Antigens and Adjuvants: Heat-killed *Mycobacterium tuberculosis* strain H37Ra

20   (Mt) was obtained from Difco. Purified protein derivative (PPD) of *M.tuber-culosis* and purified recombinant hsp65 of *M.bovis* (which is identical to *M.tuberculosis* hsp65) were kindly provided by Dr. J.D.A. van Embden, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands. Incomplete Freund's adjuvant (IFA, Difco) and dimethyl

25   dioctadecyl ammonium bromide (DDA, Eastman Kodak, Rochester, NY, ref.18) were used as adjuvants. DDA was prepared as a 20 mg/ml suspension in PBS and sonicated to produce a gel which was mixed 1:1 with Ag solution prior to immunisation.

Synthetic peptides: Peptides were prepared by automated simultaneous

30   multiple peptide synthesis (SMPS). The SMPS set-up was developed using a standard autosampler (Gilson 221) as described previously (19). Briefly, for the concurrent synthesis of peptides, standard Fmoc chemistry with Pfp-activated amino acids (Dhbt for serine and threonine) in a sixfold molar excess and Hobt as catalyst were employed. Peptides were obtained as C-

35   terminal amides from 6 mg resin/peptide (0.33 meq/g PAL resin, Millipore).

Two panels of peptides were synthesised, based on the sequences of Mt hsp65 (20) and rat hsp60 (21). Peptides were 15mers with ten amino acid overlap with each adjacent peptide (i.e residues 1-15, 6-20, 11-25 etc). Thus, every possible 11mer sequence of each protein was contained within a peptide.

Immunisations and primed lymph node populations: Rats were immunised with either Mt or hsp65. Mt was suspended at 5 mg/ml in IFA or DDA and 100 μl injected in each hind footpad (i.e. 500μg/footpad, 1 mg/rat). HSP65 (1mg/ml in PBS) was mixed 1:1 with DDA and 100 μl injected (i.e. 50 μg/footpad, 100 μg/rat). Ten to 21 days later, draining popliteal lymph nodes were removed, disaggregated, washed three times and used as a source of primed lymph node cells (PLNC). In control experiments splenocytes and lymph nodes from unimmunised rats and PLNC from rats immunised with IFA or DDA/PBS alone were used.

Immunizations with synthetic peptides and primed lymph node populations: Rats were immunized with 50μg of synthetic peptide in PBS/DDA in each hind footpad (i.e. 50 μg/footpad, 100 μg/rat). Ten days later, draining popliteal lymph nodes were removed, disaggregated, washed three times and used as a source of primed lymph node cells (PLNC). In some experiments, PLNC were derived as pooled inguinal and popliteal lymph nodes from AA rats 35-42 days post Mt immunization.

Tissue culture reagents: Iscove's modification Dulbecco's medium (IMDM, Gibco) supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco) and 5 X 10^{-5} M 2-ME was used as culture medium. Cell populations were washed in IMDM without supplements.

T cell proliferation assays: PLNC were cultured in triplicate in 200 μl flat bottom microtitre wells (Costar) at $2x10^5$ cells per well with or without antigen. In initial experiments PLNC were tested for responsiveness to individual peptides at concentrations of 5 and 20 μg/ml, and Mt, hsp65 and PPD over a range of concentrations. Concanavalin A (2 μg/ml) was used as a positive control for T cell proliferation. Cultures were incubated for 96 h at 37°C in a humidified atmosphere of 5% CO_2. Cultures were pulsed for the final 16 h with [^{3}H] TdR (Amersham, U.K., 1 μCi/well) and TdR uptake measured using a liquid scintillation β counter.

Assays using T cell lines were performed as above using $2x10^4$ line cells per well with irradiated (30 Gy) syngeneic accessory cells (either $3x10^5$ splenocytes/well or 10^6 thymocytes/well).

5   Results are expressed as mean counts per minute (cpm) of triplicate cultures. In experiments where responses to Ag were low, responses were considered significant if the stimulation index (S.I. = mean cpm with Ag − mean cpm without Ag) was greater than two and Student's t tests gave p < 0.01.

T cell lines: T cell lines with specificity for mycobacterial hsp65 or
10   peptides were generated by bulk stimulation of either hsp65-PLNC or Mt-PLNC. PLNC were cultured at $5x10^6$/ml in culture medium supplemented with 2% normal rat serum (NRS) in the presence of 10 µg/ml Ag. After three days viable cells were harvested using a ficoll-isopaque gradient and cultured for a further four days in culture medium + 5% FCS and 5% TCGF (Con A-
15   activated rat spleen supernatant). Seven days after initial stimulation lines were restimulated with irradiated spleen accessory cells and 10 µg/ml Ag in culture medium + NRS. Lines were maintained in this seven day restimulation cycle.

Short term epitope-specific T cell lines were also generated from rats
20   immunized with synthetic peptides. Peptide-PLNC were cultured as above in the presence of 10 µg/ml peptide.

Monoclonal antibodies: Anti-MHC monoclonal antibodies were added to proliferation assays to determine the MHC-restriction of responses to hsp65 and peptides. OX6 (anti-RT1.B, class II), OX17 (anti-RT1.D, class II), OX18
25   (anti-RT1.A, class I) and UD15 (anti-chloramphenicol control antibody) were used. All antibodies were mouse IgG1.

FACS Analysis: FACS analysis was used to phenotype T cell lines. Cells were incubated with either R73 (anti-αβ TCR), W3/25 (anti-CD4) or OX8 (anti-CD8), all mouse IgG1 antibodies. Second step staining was with FITC-
30   conjugated Goat anti-Mouse Ig (Becton Dickinson). Cells were analysed using a Becton Dickinson FACScan analyser.

Induction and clinical assessment of xperimental arthritis: Arthritis was induced by a single intradermal injection in the base of the tail. AA was

induced with 0.5mg Mt suspended in 100μl IFA. CP20961 arthritis was induced with 2 mg CP20961 in 100 μl light mineral oil (Sigma). Rats were examined daily for clinical signs of arthritis. Severity of arthritis was assessed by scoring each paw from zero to four based on degree of swelling, erythema
5   and deformity of the joints. Thus the maximum possible arthritis score was 16.

Modulation of arthritis with hsp65 peptides and epitope-specific T cell lines: Synthetic peptides corresponding to eight T cell epitopes present in mycobacterial hsp65 were tested for protective effects on arthritis
10   development. Rats were immunized with 100 μg of individual peptide seven days prior to arthritis induction. Peptides were immunized in each hind footpad in 10 mg/ml DDA (50 μl/pad). Control rats received only PBS/DDA. Epitope-specific T cell lines were also tested for protective activity by intravenous administration of lines at the time of arthritis induction. T
15   cell lines were restimulated *in vitro* with irradiated spleen APC and specific peptide. Four days later T cell blasts were harvested by ficoll gradient, washed and applied to a second ficoll gradient to remove any contaminating APC. T cells were washed twice in wash medium and twice in PBS and finally suspended at 2.5×10^7/ml in PBS. Immediately prior to
20   injection of Mt, 200 μl (i.e. 5×10^6) T cells were injected i.v. in the tail vein.

Results
Primed lymph node cell responses to Mt, PPD and hsp65.
    Analysis of proliferative responses revealed that naive splenocytes
25   or PLNC from rats immunised with IFA or DDA/PBS responded to some extent to whole Mt without prior immunisation (Fig. 1). These responses were not due to the presence of hsp65 which did not stimulate these populations.
    Immunisation with Mt/IFA produced increased responsiveness to Mt and also induced strong responses to PPD and low level responsiveness to hsp65,
30   indicating that Mt immunisation could prime hsp65-specific T cells.
    Immunisation with hsp65 primed for responsiveness to hsp65. PPD (which contains native hsp65) also induced proliferation indicating that immunisation with recombinant hsp65 activated T cells recognising native mycobacterial hsp65. Hsp65-PLNC showed increased responsiveness to Mt
35   compared to DDA/PBS-PLNC. Hsp65-PLNC proliferative responses to hsp65 were effectively inhibited by addition of the OX6 monoclonal antibody to

cultures, indicating that the response was predominantly (if not totally) restricted to the RT1.B^1 MHC class II molecule (Fig. 2). No significant inhibition was seen using anti-RT1.D, anti-RT1.A or anti-chloramphenicol isotype control mAbs.

5   **Identification of hsp65 T cell epitopes following immunisation with hsp65**

Hsp65-PLNC were analysed for responsiveness to a panel of peptides covering the entire sequence of hsp65 (Fig. 3a). Several peptides induced significant proliferation, suggesting the presence of seven epitopes. In terms of magnitude of response, three regions of the molecule (residues

10   176-190, 211-230 and 221-240) appeared to contain the "dominant" T cell epitopes, while regions 86-100, 251-270, 396-410 and 506-525 contained "subdominant" or minor epitopes. For four of these epitopes (211-230, 221-240, 251-270 and 506-525) responses to adjacent overlapping peptides were seen, suggesting that the core epitopes lay within 216-225, 226-235,

15   256-265 and 511-520 respectively.

Peptide 176-190 contained the AA-associated epitope 180-188. A synthetic peptide 180-188 was also tested. It was found to induce responses, but at a lower level than the longer 176-190 peptide, indicating that the minimal epitope provides less efficient stimulation at the PLNC level.

20   PLNC responses were tested at ten, 14 and 21 days post immunisation. Responses were strongest at day ten and declined with time. The pattern of dominance remained constant and no "new" responses to different peptides were observed at day 21. Initial experiments used pooled PLNC from several immunised rats. Experiments testing the responsiveness of individual rats

25   showed no variation between rats in the pattern of responsiveness. Peptides that stimulated hsp65-PLNC were also tested on unprimed splenocytes and DDA/PBS-PLNC and were found to have no stimulatory activity, indicating that immunisation with hsp65 was required to prime for the peptide induced responses.

30   Hsp65-PLNC were also tested for responses to a panel of peptides covering the entire sequence of rat hsp60. None of the rat hsp60 peptides induced significant responses in hsp65-PLNC tested at ten or 21 days post immunisation.

To confirm the presence of the hsp65 T cell epitopes identified at

35   the PLNC level, short term T cell lines were established from ten day hsp65-PLNC by bulk stimulation with hsp65. Seven days after the first restimulation, these lines were tested against the entire panel of hsp65

peptides (Fig. 3b). Surprisingly the response pattern differed from that of the original PLNC populations. The three dominant epitopes, 176-190, 216-225 and 226-235, all induced strong responses. Epitopes 256-265, 396-410 and 511-520 were also recognised, but responses to 86-105 were absent. Conversely, two peptides that failed to stimulate hsp65-PLNC (386-400 and 446-460) did stimulate the hsp65-specific lines. This alteration in response pattern following a single *in vitro* restimulation was observed in four separate experiments. None of the hsp65-specific lines showed significant responses to any rat hsp60 peptides.

T cell responses to hsp65 epitopes following immunisation with whole *M.tuberculosis*.

    Mt-PLNC were tested for responses to hsp65 peptides, to determine whether immunisation with whole Mt could prime for responsiveness to the hsp65 epitopes described above. Immunisation with Mt/IFA (i.e. the AA-inducing protocol) consistently failed to induce significant responses to hsp65 peptides (data not shown). As Mt/IFA immunisation induced only low level reactivity to hsp65 (Fig. 1), we tested PLNC from rats immunised with Mt mixed with DDA. This protocol, (using 500 µg or 1 mg of Mt per rat) produced PLNC showing increased reactivity to hsp65, and significant responses to hsp65 peptides (Figs. 1 and 4a). The response pattern differed from that obtained using hsp65-PLNC. Epitope 176-190 appeared to be dominant, epitopes 216-225, 226-235, 256-265 and 511-520 were minor and responses to epitopes 86-105 and 396-410 absent (Fig. 4a).

    Hsp65-restimulated T cell lines established from Mt/IFA-PLNC showed clear responses to hsp65 peptides (Fig. 4b), indicating that although responses to peptides could not be detected at the PLNC level, Mt/IFA immunisation did prime hsp65-specific T cells. After one *in vitro* stimulation with hsp65, epitope 176-190 was dominant, with epitopes 216-225, 226-240, 256-265 and 511-520 minor. No response could be detected to epitopes 86-100 or 396-410, suggesting that T cells specific for these epitopes are activated *in vivo* following immunisation with hsp65 but not whole Mt. Lines restimulated with Mt showed an even stronger dominance of the response to epitope 176-190. In contrast to hsp65-restimulation, responses to epitope 256-265 were absent. Responses to peptides 386-400 and 446-460 were detected in lines from Mt immunised rats after restimulation with hsp65 but not Mt. Lines from Mt/DDA-PLNC showed identical response patterns.

Analysis of T cell lines specific for defined hsp65 epitopes

T cell lines were generated by restimulation of hsp65-PLNC with individual synthetic peptides. For epitopes where responses had been detected to two overlapping peptides, the line was generated using the
5   peptide that induced strongest proliferation of hsp65-PLNC (e.*g.* for epitope 216-225, peptide 211-225 was used). This resulted in eight T cell lines. Table I summarises the response patterns to each epitope and names each peptide-specific T cell line. No lines specific for peptide 386-400 were established.
10   After at least four *in vitro* restimulations each line was tested for responsiveness to specific peptide, hsp65, Mt and PPD. To test for cross-reactivity with self hsp60, each line was also tested against the corresponding rat hsp60 peptide (Fig. 5).

All lines responded to hsp65 and their specific peptides and, in all
15   but one instance, an overlapping peptide. Between residues 211 and 235 four overlapping peptides were stimulatory. Thus it was possible that four separate epitopes were present in this region. However, T cell lines generated against peptides 216-230 and 221-235 responded more strongly to peptides 211-225 and 226-240 respectively, indicating the presence of only
20   two epitopes: 216-225 and 226-235 as described above. Line H.36, specific for peptide 176-190 also responded to a peptide including residues 180-188, previously described to be recognised by the arthritogenic T cell clone A2b, indicating that these two lines recognise the same core epitope.

While all lines responded to Mt to some extent, the level of pro-
25   liferation varied between lines. Lines H.36 and H.46 both showed strong responses to Mt, while the other six lines responded poorly. All lines responded to PPD.

Line H.52, specific for residues 256-265, showed clear responses to the homologous peptide of rat hsp60. Therefore, while no responses to rat
30   hsp60 peptides could be detected using hsp65-PLNC or the hsp65-specific lines, cross-reactive T cell recognition could be demonstrated using this epitope-specific cell line. The mycobacterial peptide induced a five to ten fold higher level of proliferation than the rat peptide. This might explain why the rat peptide was not recognised at the PLNC level as responses to
35   the mycobacterial peptide were minor and any decrease in stimulatory activity could result in responses below the detectable level. The core epitope recognised by H.52 contains only three residues which differ from the rat hsp60 sequence, situated at the C terminal end of the epitope (see

Table I). All other T cell lines failed to respond to rat hsp60 peptides. Thus, of the nine T cell epitopes in mycobacterial hsp65 identified by this study, only one showed cross-reactivity with rat hsp60.

5   All peptide-specific or hsp65-specific T cell lines were analysed for T cell phenotype and MHC-restriction. FACS analysis showed that all T cell lines were $\alpha\beta$ TCR$^+$ CD4$^+$ CD8$^-$. MHC-restriction was determined by assessing the ability of anti-MHC monoclonal antibodies (10 μg/ml) to inhibit hsp65 or peptide (1 μg/ml) induced responses in proliferation assays. Addition of anti-RT1.B reduced proliferation of all lines by
10 greater than 70%, while anti-RT1.D or anti-RT1.A had no significant effect (Fig. 6). Thus all lines were RT1 B^1-restricted.

Immunization with hsp65 peptides primes for epitope-specific T cell responses

    To determine whether immunization of rats with synthetic peptides
15 was effective at priming for T cell reactivity to hsp65 epitopes, we examined *in vitro* proliferative responses following immunization with 100μg of peptide each containing individual T cell epitopes. PLNC isolated ten days after immunization were tested for responsiveness to the immunizing peptide, overlapping peptides and to hsp65 (Table II). PLNC responses were
20 observed after immunization with seven of the nine peptides. Responses were not seen after immunization with peptides 386-400 and 511-525.

    Bulk stimulation generated T cell lines to eight of the nine peptides tested. These eight lines were tested for specificity after four *in vitro* restimulations (Table II). All responded to the immunizing peptide
25 and, to some extent, to hsp65. Also, the lines were tested for responsiveness to overlapping peptides and, in all but one case, showed identical response patterns to T cell lines generated previously against the same epitopes after immunization with whole hsp65. These findings suggest that T cells activated *in vivo* by immunization with hsp65 or synthetic peptides
30 recognize the same core epitopes.

    A T cell line specific for th 86-100 peptide generated from rats immunized with hsp65 responded to peptides 86-100 and 91-105 (i.e. recogned a core epitope of 91-100). The T cell line generated following immunization with the 86-100 peptide also responded to peptides 81-95 and 91-105. This
35 suggested the presence of two T cell populations, one recognising residues 86-95, the other recognising residues 91-100. Interestingly, the sequences of mycobacterial hsp65 and rat hsp60 covering residues 86-95 are identical.

Accordingly, the line proliferated in response to peptides corresponding
to rat hsp60 81-95 and 86-100. Thus immunization with the hsp65 86-100
peptides could prime for responsiveness to an epitope in self hsp60.

5   Line H.52 (generated from hsp65-immunized rats and recognizing
epitope 256-265) also responded to the highly homologous rat hsp60 256-270
peptide (see above). Accordingly the 256-270 specific T cell line derived
from peptide immunized rats also responded to rat 256-270 (Table II). This
line also showed an increased "autoreactive" response to syngeneic APC
which had been heat-shocked (one hour at 42°C prior to irradiation) in
10   comparison control APC cultured at 37°C (Table III). This suggested that
increased expression of endogenous hsp60 by APC results in presentation of
this cross-reactive epitope in association with MHC for T cell recognition.

Analysis of the ability of hsp65 peptides to vaccinate against arthritis

        The effects of preimmunization with synthetic peptides containing
15   individual epitopes on the development of AA were analysed (Fig. 7). The
eight peptides which primed for epitope specific T cells were tested. Rats
were immunized with 100µg of peptide seven days prior to AA-induction with
Mt.

        Preimmunization with peptide 256-270 resulted in clear protective
20   effects against AA development. The mean maximum arthritis score after 256-
270 preimmunization was 1.7 (24 rats in five separate experiments) compared
to mean maximum score of 11.5 for control rats preimmunized with PBS. Of
the 24 rats preimmunized with 256-270, twelve did develop clinical signs
of arthritis, which were milder than those developed by control rats. Also,
25   whereas control rats suffered permanent joint deformities that persisted
after the initial arthritis had subsided, the 256-270 preimmunized rats
that developed AA were remarkably free of permanent deformities. Pre-
immunization with the other peptides containing hsp65 T cell epitopes had
no significant effects on the onset of AA.

30   PLNC from preimmunized rats were tested for responsiveness to hsp65
epitopes 42 days after Mt administration (Fig. 8). PLNC from rats pre-
immunized with PBS/DDA alone showed significant responses to peptide 176-
190 (containing the AA-associated 180-188 epitope) but not to any other of
the defined hsp65 epitopes. PLNC from rats preimmunized with individual
35   hsp65 peptides all showed responses to the immunizing peptide, although the
level of proliferation varied. Thus, whereas preimmunization with peptides
211-225 and 446-460 induced strong responses, preimmunization with peptide

256-270 (which protected against AA) induced marginal PLNC responses. Responses to peptide 176-190 containing the AA-associated epitope were found in all PLNC populations (and enhanced after 176-190 preimmunization) with one exception. PLNC from rats protected from AA by preimmunization
5   with peptide 256-270 showed a total lack of response to 176-190.

Administration of a T cell line specific for the cross-reactive 256-265 epitope confers protection against AA

Preimmunization with hsp65(256-270) protected against arthritis. To determine whether this activity was the result of activation of T cells
10   specific for this epitope, the effects of epitope-specific T cell lines on AA were tested (Fig. 9). T cell lines were administered to rats at the same time as Mt for AA induction (5×10^6 T cells per rat injected i.v. in a tail vein). In two experiments, administration of line H.52 recognising the 256-265 epitope clearly reduced the severity of AA compared to control animals
15   receiving PBS. The T cell lines H.36, H.43 and H.46 (specific for epitopes 180-188, 216-225 and 226-235 respectively) were also tested and had no significant effects on AA development.

Line H.46 was restimulated with specific peptide (226-240) in the presence of 10 µg/ml peptide 256-270, in order to ensure that the
20   protective effect of line H.52 was not the result of administration of residual 256-270 peptide carried over from the *in vitro* restimulation of the line. Administration of the resulting 226-235 specific T cell blasts failed to protect against AA.

Preimmunization with peptide 256-270 also vaccinates against CP20961-
25   **induced arthritis**

The only hsp65 peptide which protected strongly against AA was found to be 256-270, which induced T cell reactivity against the corresponding sequence of rat hsp60. This finding suggested that self-reactive T cells account for hsp65-induced protection against arthritis. This mechanism
30   would then be expected to protect against arthritis induced by other compounds and not be dependent on the use of mycobacteria. Therefore the capacity of peptide 256-270 to induce protection in the CP20961-induced model was tested. As CP20961 is a lipoidal amine, there is no possibility of this arthritogenic preparation containing an antigenic component with
35   potential cross-reactivity with hsp65 256-270. Preimmunization with hsp65 256-270 strongly protected rats against CP20961-induced arthritis, whereas

a control peptide (hsp65 211-225) did not (Fig. 10). Thus preimmunization with hsp65 256-270 can protect against AA and CP20961-induced arthritis, presumably via a mechanism which is not dependent on the administration of mycobacteria as a component of the arthritogenic inoculation.

5   **Rat hsp60(256-270) fails to vaccinate against arthritis**

Since preimmunization with hsp65 256-270 provided protection against AA and T cells specific for this peptide also responded to the highly homo-logous peptide based on the rat hsp60 256-270 sequence, the rat peptide was tested for similar protective effects (Fig. 11). Protection was observed

10   after preimmunization with mycobacterial hsp65 256-270, but not with rat hsp60 256-270. An explanation for this discrepancy may be that immunization with rat hsp60 256-270 does not prime for T cell responses. To investigate this we generated peptide-specific T cell line from rat 256-270 (R.256-270) immunized PLNC and compared responses of this line with that of a line from

15   mycobacterial 256-270 (M.256-270) immunized PLNC (Fig. 12). The line generated against M.256-270 responded to peptides representing M.256-270, M.251-265 and a shorter "core" peptide M.256-265, and cross-reacted with R.256-270 and (weakly) R.256-265. In contrast, the line generated against R.256-270 responded to R.256-270, but not to M.256-270 or to R.256-265 and

20   M.256-265. This line also showed a high "autoreactive" response to APC alone, suggesting that the APC were already expressing the self hsp60 epitope.

Thus immunization with M.256-270 primed for T cell responses that cross-reacted with R.256-270, but immunization with R.256-270 primed for

25   rat-specific T cell responses that did not cross-react with M.256-270. The fact that the R.256-270 specific line did not respond to R.251-265 or R.256-265 suggests that one or more of the five C-terminal residues of R.256-270 is important for stimulation of this line. None of these five residues share identity with the mycobacterial sequence, presumably

30   accounting for the lack of responsiveness to M.256-270.

Furthermore, using anti-MHC mAbs, the T cell lines generated against M.256-270 and R.256-270 were found to have different MHC-restrictions. Peptide induced proliferation of the M.256-270 specific line was inhibited by the OX6 anti-RT1.B mAb, whereas the R.256-270 specific line was in-

35   hibited by the OX17 anti-RT1.D mAb.

Table I

Mycobacterial hsp65 epitopes recognised by Lewis rat T cells.

Immunisation		hsp	hsp	Mt	Mt	Mt	hsp	
In vitro stimulation		-	hsp	-	hsp	Mt	peptide	
Epitope	Sequence[1]							T cell line
91-100	DGTTTATVLAQALVR	+	-	-	-	-	+[2]	H.18
176-190	EESNTFGLQLELTEG	+++	+++	+++	+++	+++	+	H.36
216-225	AVLEDPYILLVSSKV	+++	+++	+	+	+	+	H.43
226-235	STVKDLLPLLEKVIG	+++	+++	+	+	+	+	H.46
256-265	ALSTLVVNKIRGTFK	+	+	+	+	-	+	H.52
386-400	ELKERKHRIEDAVRN	-	+	-	+	-	-	-
396-405	DAVRNAKAAVEEGIV	+	+	-	-	-	+	H.80
446-455	APLKQIAFNSGLEPG	-	+	-	+	-	+	H.90
511-520	FLTTEAVVADKPEKE	+	+	+	+	+	+	H.103

Footnotes
1. The sequences of hsp65 peptides used to generate each line are shown.
Core epitopes, as defined by responses to overlapping peptides, are denoted
by underlined residues. Residues sharing identity with the corresponding
sequence of rat hsp60 are in bold.
Differential recognition of epitopes following differing immunisation and
restimulation protocols are summarised.
-         No response
+   Minor response
+++   Dominant response
2. + and - in this column refer to whether a peptide-specific T cell line
was generated from hsp65-PLNC.

Table II

Immunization with hsp65 peptides primes for T cell responses.
Rats were immunized with synthetic peptides containing individual hsp65
epitopes (100μg peptide/DDA per rat). Ten days later PLNC were isolated and
tested for responses to overlapping peptides (20 μg/ml). Peptide-specific
T cell lines were generated by bulk in vitro stimulation of PLNC with
immunizing peptide. Lines were tested for responses to overlapping peptides
(10 μg/ml). All PLNC and T cell lines showed significant responses to 20
μg/ml hsp65 (data not shown). Results are expressed as mean cpm of tripli-
cate cultures. All SEM were less than 20%.

Table II

Immunizing peptide	In vitro peptide	CPM		Response after hsp65 immunization[1]
		PLNC	T Cell Line	
86-100	0	2518	1127	
	81-95	15296	15785	-
	86-100	57925	49422	+
	91-105	1999	18444	+
	Rat 86-100	6196	25535	-
176-190	0	1784	1255	
	171-185	3082	1640	-
	176-190	57707	31400	+
	181-195	1916	1653	-
	180-188	7859	23275	+
211-225	0	986	1479	
	206-220	645	988	-
	211-225	39765	121978	+
	216-230	11117	102341	+
226-240	0	1286	882	
	221-235	7381	100306	+
	226-240	22761	152071	+
	231-245	1655	862	-
256-270	0	2448	762	
	251-265	6391	66063	+
	256-270	16423	69037	+
	261-275	2553	646	-
	Rat 256-270	4152	14987	+
396-410	0	3399	2095	
	391-405	2280	39335	+
	396-410	8477	88916	+
	401-415	3211	2011	-
446-460	0	1928	1028	
	441-455	4715	17805	+
	446-460	22918	38324	+
	451-465	2130	1283	-
511-525	0	2804	537	
	506-520	2754	61293	+
	511-525	3164	129373	+
	516-530	2821	1231	-

[1]. As determined using T cell lines generated from hsp65-immunized rats.

Table III

Heat-shocked APC stimulate hsp65(256-270) specific T cells.
T cell lines (2x10⁴/well) were cultured with APC (2x10⁵/well) that had been
cultured for one hour at either 37°C or 42°C prior to irradiation. Cells
were cultured with or without specific peptide as Ag (10μg/ml). Line H.43,
specific for the non-cross-reactive, mycobacterial hsp65 unique, epitope
211-225 was used as a control. Results are expressed as mean cpm of
triplicate cultures. All SEM were less than 20%.

T cell line	P.m52.1	Line H.46
Specificity	256-265	226-235
T cells - APC	44	22
37°C APC - Ag	470	33
+ Myco. pept	120744	162785
+ rat pept	18061	NT
42°C APC - Ag	15960	37
+ Myco. pept	115626	150887
+ rat pept	25842	NT

Discussion

Immunisation of Lewis rats with heat-killed Mt in IFA induces AA
(1), reported to be associated with T cell responses to residues 180-188
of mycobacterial hsp65 (5). Conversely, immunisation with hsp65 protects
against subsequent attempts to induce AA by an as yet undefined T cell-
mediated mechanism (5,6). Epitopes within hsp65 recognised by Lewis rat T
cells were identified in this description. Responsiveness to these epitopes
in T cell populations following immunisation with either Mt or hsp65 was
compared.

Immunisation of rats with recombinant mycobacterial hsp65 primed
for MHC class II (RT1.B¹)-restricted recognition of hsp65. Analysis of
hsp65-PLNC responses to overlapping peptides covering the entire hsp65
sequence identified seven T cell epitopes. Following a single *in vitro*
restimulation with hsp65, two further epitopes were identified. Thus Lewis
rat T cells recognise nine epitopes in mycobacterial hsp65.

Immunisation with Mt suspended in IFA (as used in the AA-inducing
protocol) did not prime for PLNC responses to hsp65 peptides. However,
Mt/DDA immunisation did induce significant responses to hsp65 peptides.

Significantly, AA only develops following Mt/IFA immunisation and not after
Mt/DDA immunisation (S.M.A, unpublished observations). Thus the enhanced
activation of hsp65-specific T cells when DDA is used as adjuvant might
account for this difference. T cells from Mt/IFA and Mt/DDA immunised rats
5   showed identical response patterns after *in vitro* restimulation with hsp65
indicating that Mt/IFA does prime for T cell recognition of hsp65, but at
a relatively low level.

Patterns of dominance of hsp65 epitopes differed following
immunisation with hsp65 or whole Mt. Hsp65 immunisation resulted in three
10 co-dominant epitopes: 176-190, 216-225 and 226-235. After Mt immunisation,
epitope 176-190 appeared dominant with epitopes 216-225 and 226-235 being
minor along with 256-265 and 511-520. The dominance of the 176-190 epitope
was even more marked when Mt-PLNC were restimulated with Mt. Hsp65, but not
Mt immunisation primed for responses to epitopes 86-100 and 396-410.

15 We generated αβ TCR⁺CD4⁺, RT1.B¹-restricted T cell lines specific for
eight of the nine epitopes identified. Of these eight T cell lines two,
H.36 and H.46 (which recognise 176-190 and 226-235 respectively) responded
strongly to Mt while the others responded relatively weakly. This is
consistent with the dominance of 176-190 following immunisation with Mt.

20 The relatively low quantities of hsp65 present in the Mt preparation
might result in focussing of T cell responses on hsp65 epitopes with higher
affinities for MHC class II molecules. Preliminary experiments testing the
ability of peptides containing hsp65 epitopes to inhibit proliferation of
T cell lines with other Ag specificities suggest that peptide 176-190 may
25 have a higher affinity for RT1.B¹ molecules than do peptides 211-225 or
226-240 (data not shown). Thus, when Ag concentrations are limited (i.e.
following Mt immunisation) dominance of T cell epitopes might be more
dependent on their relative MHC binding affinities, whereas following
immunisation with large quantities of specific antigen (50-100 µg hsp65 per
30 rat) relative dominance might not be simply a function of affinity for MHC.
Clearly, antigen processing of the intact hsp65 molecule will determine the
peptide fragments generated during *in vivo* priming, and the naturally
processed fragments will not be identical to the synthetic 15mers used in
this study. Also the molecular context of hsp65 (i.e recombinant monomeric
35 hsp65, or the multimeric native protein in the Mt preparation) could affect
antigen processing and influence the pattern of epitope recognition.

Significantly, the dominant hsp65 pitope following Mt immunisation,
176-190, contains the 180-188 sequence previously reported to be recognised

by the arthritogenic T cell clone A2b. Therefore, the AA-inducing protocol results in a T cell response skewed towards the AA-associated epitope. For PLNC responses, the longer 176-190 peptide induced stronger proliferative responses than the minimal 180-188 peptide. This is a significant finding
5 as previous studies have analysed polyclonal responses to 180-188 (7,22). The length of naturally processed peptides found in the binding groove of MHC class II molecules has been described as 13 to 25 amino acids (23,24). Thus a more stable interaction of the 15mer 176-190 with the RT1.B[1] molecule compared with the 9mer 180-188 might account for the increased
10 stimulatory activity.

Of the nine hsp65 T cell epitopes defined by this study, one was cross-reactive with rat hsp60. Although no significant response to any rat hsp60 peptide was observed at the PLNC level following immunisation with either hsp65 or Mt, the T cell line H.52, specific for the 256-265 epitope
15 did respond to the corresponding rat hsp60 peptide. Accordingly this region is highly conserved (mycobacterial: ALSTLVVNKI, rat: ALSTLVLNRL) with seven residues identical and conservative substitutions at the other three positions. The region 243-265 shows highest identity between mycobacterial and mammalian hsp60s, with 18/23 residues identical and five conservative
20 substitutions (11).

The present findings provide important insights into T cell mediated protective effect of hsp65 preimmunisation in experimental models of arthritis, for which three possible mechanisms have been proposed. Firstly, previous data suggested that hsp65 preimmunisation might down-regulate the
25 response to the AA-associated 180-188 epitope (22). The results of the present study do not support this, as 176-190 is a co-dominant epitope following hsp65 immunisation.

Secondly, enhanced activation of T cells (recognising one or more hsp65 epitope) following preimmunisation might result in a more efficient
30 recognition and clearance of Mt on subsequent challenge, before AA can develop. It was found that hsp65-immunisation results in improved recognition of hsp65 epitopes compared to Mt-immunisation (both in terms of number of epitopes recognised and magnitude of responses). These differences might form the basis for such a protective mechanism.
35 Reports of T cell recognition of epitopes conserved between myco-bacterial hsp65 and mammalian hsp60 (14-17) have led to a third hypothesis, in which preimmunisation with hsp65 activates T cells recognising cross-reactive epitopes (16). Subsequent recognition of self hsp60 upregulated

during inflammation within the joint would then regulate the inflammatory process, preventing development of chronic arthritis. If this hypothesis is correct, the relevant T cell epitope (for models using Lewis rats) must be residues 256-265 as this is the only cross-reactive epitope recognised following hsp65 immunisation. Interestingly, epitope 256-265 was recognised only poorly by Mt/IFA immunisation. Also, line H.52 responded weakly to Mt. Therefore the AA-inducing protocol is poor at activating T cells specific for this epitope.

Preimmunisation with hsp65 not only protects against AA, but also against arthritis induced without mycobacteria (6-10) and, in pristane (10) and CP20961-induced (6) arthritis, without any exogenously added protein. Therefore it is probable that the pathogenic mechanisms in these models differ, yet all can be prevented by preimmunisation with hsp65. With this in mind, the hypothesis which does not require recognition of whole Mt, but involves cross-reactive T cell recognition of mycobacterial hsp65 and rat hsp60 seems most attractive as protection could be accounted for by a single mechanism, regardless of the arthritogenic agent used. T cell recognition of self hsp60 might also be relevant in resistance to human arthritic conditions. T cell reactivity to self hsp60 has been reported in patients with RA (25) and JCA (26). An exciting extension of this was the report of two CD4⁺ T cell clones from a JCA patient which recognised cross-reactive epitopes in the highly conserved region (243-265) recognised by the H.52 T cell line in our study, and that the donor patient had a favourable outcome of disease (17). Also epitopes in this region of self hsp60 were recognised by CD4⁺ CTL from healthy human donors (14).

T cell reactivity against hsp65 is believed to be involved in the protective mechanism(s). Rats were preimmunized with synthetic peptides containing individual epitopes seven days prior to AA induction with Mt. This approach led to a striking protective effect in rats preimmunized with peptide 256-270. None of the other peptides tested showed any influence on AA development. The T cell line H.52, originally generated from hsp65 immunized rats and specific for epitope 256-265, also showed a protective effect on AA development when injected i.v. at Mt administration. Transfer of line H.52 did not induce total protection against AA but clearly reduced the severity of the arthritis. The low numbers of cells transferred in this study (5×10^6 compared with 5×10^7-10^8 in other studies), enforced due to the slow rate of growth displayed by H.52, might be insufficient to induce a fully protective effect.

The finding that preimmunization with peptide 176-190 (containing the AA-associated 180-188 epitope) had no effect on AA onset is in contrast with previous reports that preimmunization with peptides 180-188 induces effective protection against AA. A possible explanation for this difference lies in the different preimmunization regimens adopted. We immunized with peptide in the footpads seven days prior to Mt immunization using DDA as adjuvant, whereas the previous studies immunized i.p. with peptide in IFA on days -35, -20 and -5. Although i.p. immunization with peptide in IFA has been used to induce epitope specific T cell tolerance, this approach was reported to induce 180-188 specific T cells capable of transferring protection to naive rats. These findings could not be repeated.

No protective effect was found of administering the 180-188-specific T cell line H.36 at the time of Mt immunization. This contrasts with the previously described protective effects of transferring spleen T cells from 180-188 immunized rats or the 180-188 specific T cell lines A2 and A2c. H.36 might therefore be an "A2b-like" line with AA inducing rather than protective activity, although transfer of H.36 did not increase severity of AA and we have not tested the ability of the line to induce AA in irradiated rats. Alternatively, the low numbers of cells transferred in this study again might not be sufficient to induce a protective effect.

Line H.52 has been shown to recognize the 256-270 sequence of rat hsp60. Similarly, short term T cell lines derived from rats immunized with peptide 256-270 (generated both from rats ten days after peptide immunization and from protected rats 42 days after Mt administration) responded to the corresponding rat hsp60 peptide. These lines also showed small but significant responses to heat shocked APC indicating that the endogenous self hsp60 epitope could be presented in association with MHC class II for T cell recognition when hsp60 levels were upregulated.

Thus, immunization with mycobacterial hsp65 256-270 protected against AA development and activated T cells capable of responding to the shared epitope in rat hsp60. This finding provides support for the hypo-thesis that the mechanism by which hsp65 preimmunization protects Lewis rats against arthritis is based on activation of T cells that recognize an epitope shared with rat hsp60. Recognition by these T cells of elevated levels of the self epitope presented by MHC class II expressing cells at the site of inflammation (the joint) would then provide an antigen-specific mechanism for regulation of the inflammatory process. This mechanism does not require the "protective" T cell to recognize a mycobacterial component

and therefore provides an attractive single mechanism for explaining hsp65-induced resistance to models not employing bacterial derived-arthritogens. The most notable of these models in the Lewis rat is the model induced with CP20961, which is a lipoidal amine and therefore has no possible antigenic cross-reactivity with hsp65. It was found indeed that peptide 256-270 confers protection against the CP20961-induced model.

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Description of the figures

Figure 1: Mt or hsp65 immunisation primes for T cell recognition of hsp65.

Ten day PLNC from rats immunised with hsp65, Mt/IFA, or Mt/DDA were tested

against hsp65, PPD and Mt over a range of concentrations (20 µg/ml, which

gave maximal responses, shown here). Unimmunised splenocytes and PLNC from

rats immunised with PBS/DDA were used as negative controls (PLNC from IFA

immunised rats showed the same response pattern as PBS/DDA-PLNC). All

S.E.M's were less than 20%.

Figure 2: Anti-MHC mAb inhibition of anti-hsp65 PLNC responses.

Ten day hsp65-PLNC were cultured with a range of hsp65 doses in the

presence of 10 µg/ml mAb specific for RT1.B (OX6), RT1.D (OX17) and RT1.A

(OX18). Anti-cmp = UD15 anti-chloramphenicol, isotype control antibody. All

S.E.M's were less than 20%.

Figure 3: Responses to mycobacterial hsp65 peptides following immunisation

with hsp65.

Responses to the entire panel of hsp65 peptides were analysed using ten day

hsp65-PLNC (a), or an hsp65-specific T cell line (b), generated from the

same hsp65-PLNC population. Peptides were tested at 5 and 20 µg/ml (20

µg/ml shown here). Responses to hsp65, PPD and Mt (20µg/ml) were all

greater than 60,000 cpm in (a) and 150,000 cpm in (b). Peptides inducing

significant responses are identified. Where overlapping peptides were

stimulatory, the peptide giving stronger responses is identified. The

results of this experiment were reproduced in three further experiments.

All S.E.M's were less than 20%.

Figure 4: Responses to mycobacterial hsp65 peptides following immunisation
with whole Mt.
Responses to the entire panel of hsp65 peptides were analysed using ten day
5   PLNC from rats immunised with Mt/DDA (a), or T cell lines generated from
Mt/IFA-PLNC by restimulation with hsp65 or Mt (b). Peptides were tested at
5 and 20 μg/ml (20 μg/ml gave stronger responses, and is shown here).
Responses to hsp65, PPD and Mt (20μg/ml) were all greater than 40,000 cpm
in (a) and 150,000 cpm in (b). Peptides inducing significant responses are
10   highlighted. All S.E.M's were less than 20%.

Figure 5: Response of T cell lines specific for defined hsp65 epitopes.
T cell lines were tested for responses to overlapping hsp65 peptides,
corresponding rat hsp60 peptides, hsp65 and Mt over a range of Ag
concentrations (10 μg/ml shown here). All lines responded to PPD and none
15   responded to control hsp65 peptides containing other epitopes (data not
shown). All S.E.M's were less than 20%.

Figure 6: Anti-MHC mAb inhibition of T cell line responses.
Epitope-specific and hsp65-specific T cell lines were cultured with irradi-
ated APC and 1 μg/ml Ag (specific peptide for epitope-specific lines or
20   hsp65 for the hsp65-specific line) in the presence of 10 μg/ml mAb specific
for RT1.B (OX6), RT1.D (OX17) and RT1.A (OX18). Anti-cmp = UD15 anti-
chloramphenicol, isotype control antibody. All S.E.M's were less than 20%.

Figure 7: Modulation of AA development by preimmunisation with hsp65
peptides.
25   Rats were immunized in the hind footpads with 100 μg of individual
synthetic peptides or PBS in DDA seven days prior to AA induction using 5mg
Mt in 100μl IFA injected i.d. at the base of the tail. Five rats were used
in each preimmunization group. Arthritis scores were assessed daily from
eight days after Mt injection.

30   Figure 8: PLNC responses of peptide-preimmunized AA rats.
Rats were preimmunizaed and Mt-immunized as described in Fig. 7. PLNC
(pooled inguinal and popliteal LN) were isolated 42 days after Mt-
immunization and tested for responses to hsp65 paptides containing defined

T cell epitopes (20μg/ml). All PLNC responded to hsp65, Mt (responses were all greater than 50,000 cpm). All SEM were less than 20%.

Figure 9: Modulation of AA using epitope-specific T cell lines.
Rats were administered with 5×10^6 T cells i.v. in PBS or PBS alone at the time of AA induction using 5mg Mt in 100μl IFA injected i.d. at the base of the tail. Five rats were used in each group. Arthritis scores were assessed daily from eight days after Mt injection. Results using lines H.46 (specific for 226-235) and H.52 (specific for 256-265) are shown. Injection of lines H.36 and H.43 (specific for epitopes 180-188 and 216-225 respectively) had no significant effect on AA (data not shown).

Figure 10: Modulation of CP20961-induced arthritis by preimmunization with hsp65 peptides.
Rats were immunized in the hind footpads with 100 μg of individual synthetic peptides (211-225 or 256-270) or PBS in DDA seven days prior to AA induction using 2mg CP20961 in 100μl mineral oil injected i.d. at the base of the tail. Five rats were used in each preimmunization group. Arthritis scores were assessed daily from eight days after Mt injection.

Figure 11: Preimmunization with rat hsp60(256-270) does not protect against AA
Rats were immunized in the hind footpads with 100 μg of individual synthetic peptides or PBS in DDA seven days prior to AA induction using 5mg Mt in 100μl IFA injected i.d. at the base of the tail. Five rats were used in each preimmunization group. Arthritis scores were assessed daily from eight days after Mt injection. Preimmunization with a control peptide (hsp65 211-225) did not influence AA development (data not shown).

Figure 12.
Immunization with hsp65(256-270) and rat hsp60(256-270) primes for T cell responses to distinct epitopes. Rats were immunized in the hind footpads with either M.256-270 or R.256-270 in DDA. Seven days later PLNC were isolated and restimulated *in vitro* with the immunized peptide. The resulting T cell lines (line P.m52 recognizing M.256-270, and line P.r57 recognizing R.256-270) were tested for responses to mycobacterial and rat peptides in the presence of irradiated syngeneic spleen APC. All SEM were less than 20%.

Claims

1.   Polypeptide containing a part of the amino acid sequence of the heat shock protein hsp65 of *Mycobacterium tuberculosis* as depicted in SEQ ID No. 1, comprising at least 5 aminoacids which are in the same relative
5   position as the same aminoacids in a T cell epitope of hsp65 that contains at least 4 consecutive aminoacids which are identical with the corresponding mammalian hsp60 aminoacids.

2.   Polypeptide according to claim 1, wherein the polypeptide comprises at least 5 aminoacids which are in the same relative position as the same
10   aminoacids in one of the sequences 81-100 and 241-270 of SEQ ID No. 1.

3.   Polypeptide according to claim 2, wherein the polypeptide comprises at least 5 aminoacids which are in the same relative position as the same aminoacids in one of the sequences 84-95 and 256-265 of SEQ ID No. 1.

4.   Polypeptide according to any one of claims 1-3, wherein the poly-
15   peptide comprises 5-30 aminoacids of the amino acid sequence of hsp65.

5.   Polypeptide analogue which exhibits the immunological properties of a peptide according to any one of claims 1-4, but which contains one or chemical modifications.

6.   Nucleotide sequence encoding a polypeptide according to any one of
20   claims 1-4.

7.   Expression system capable of expressing a polypeptide according to any one of claims 1-4.

8.   Microorganism containing an expression system according to claim 7.

9.   Eukaryotic cell containing an expression system according to
25   claim 7.

10.   Cell expressing a receptor from a T cell activated by immuno-stimulation using a polypeptide according to any one of claims 1-5.

11.   Antibody raised against a polypeptide according to any one of claims 1-5.

12.   Pharmaceutical composition suitable for protection against or treat-ment of an inflammatory disease, including autoimmune diseases, diabetes, arthritide diseases, atherosclerosis, multiple sclerosis, myasthenia gravis, containing a polypeptide according to any one of claims 1-5, a nucleotide sequence according to claim 6, an expression system according to claim 7, a cell according to any one of claims 8-10, or an antibody according to claim 11.

13.   Diagnostic composition containing a polypeptide according to any one of claims 1-5 or an antibody according to claim 11.

Abstract

Polypeptides are provided which are useful for protection against or treat-
ment of an inflammatory disease, including autoimmune diseases, diabetes,
arthritide diseases, atherosclerosis, multiple sclerosis, myasthenia
5   gravis. The polypeptides contain a part of the amino acid sequence of the
heat shock protein hsp65 of *Mycobacterium tuberculosis* as depicted in
SEQ ID No. 1, comprising at least 5 aminoacids which are in the same
relative position as the same aminoacids in a T cell epitope of hsp65 that
contains at least 4 consecutive aminoacids which are identical with the
10 corresponding mammalian hsp60 aminoacids. Preferred T cell epitopes are
located within the sequences 81-100 and 241-270 of SEQ ID No. 1.
Nucelotide sequences, expression systems, antibodies and pharmaceutical and
diagnostic compositions derived from thee polypeptides are provided as
well.

Figure 1
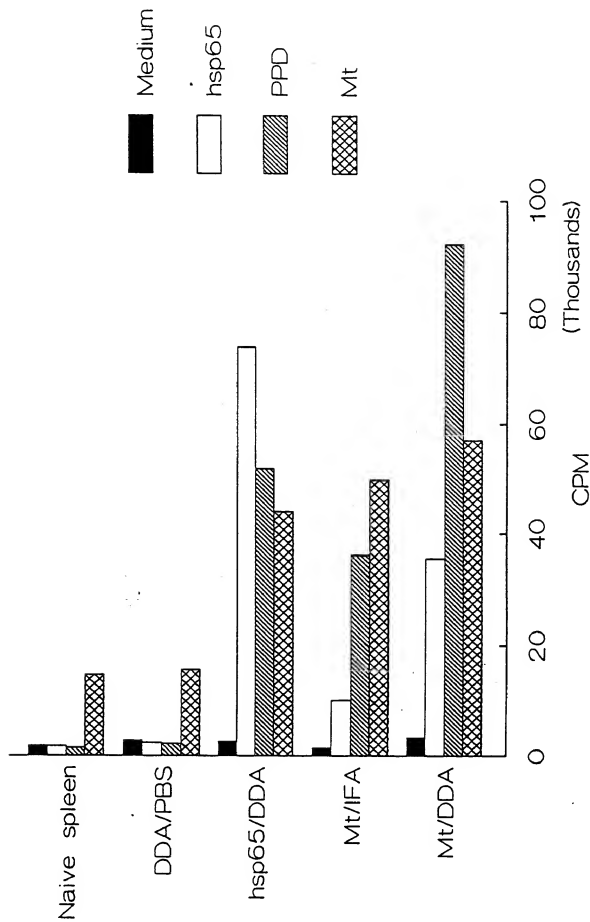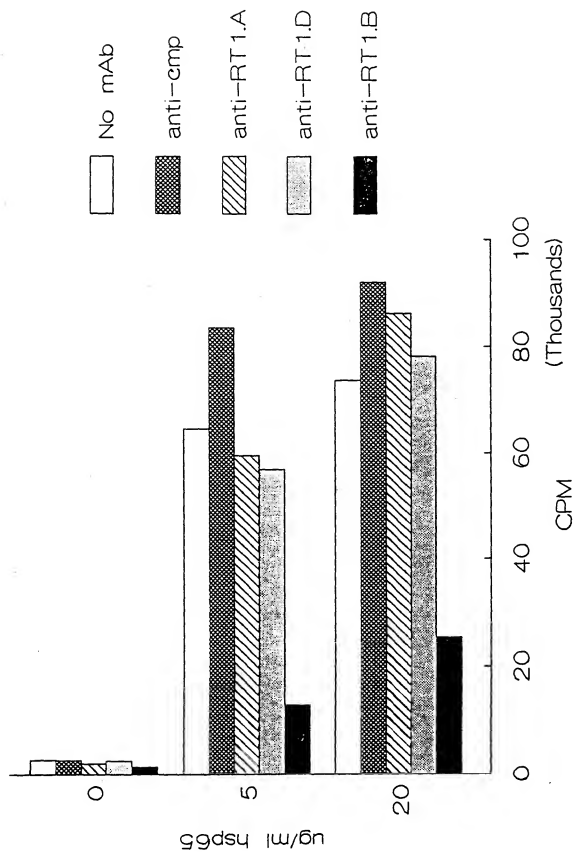
Figure 2

Figure 3
A. Hsp65-PLNC
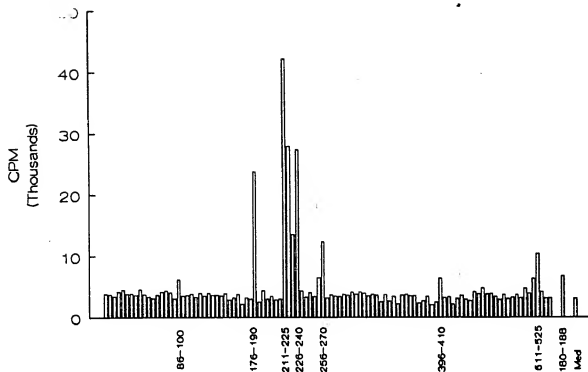
B. Hsp65-specific T cell line
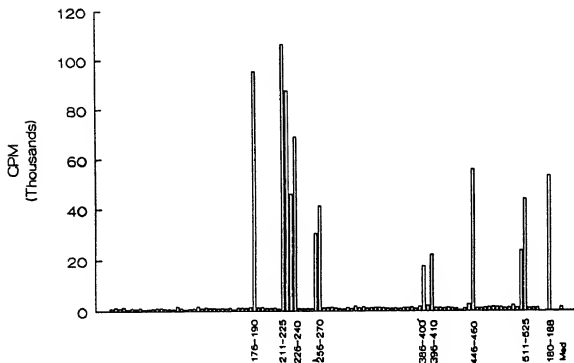
Figure 4

A. Mt/DDA-PLNC
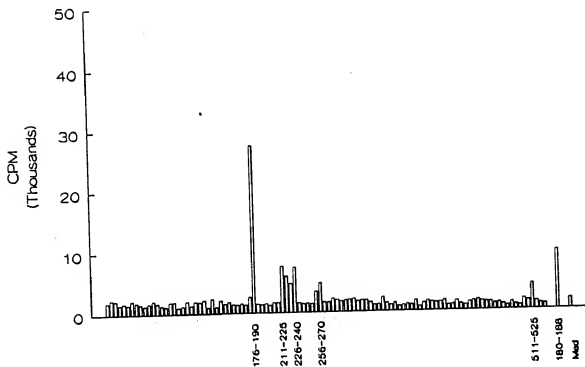
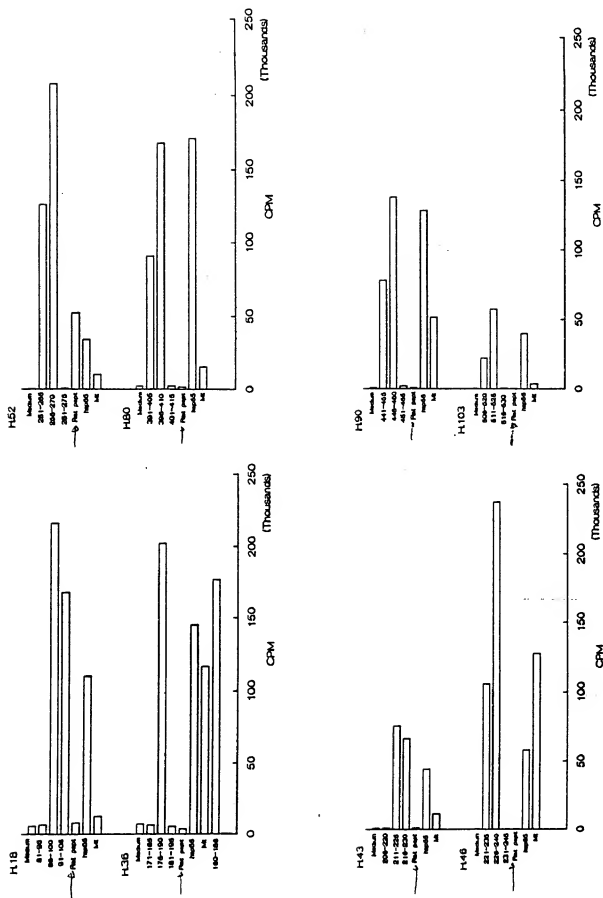B. Mt/IFA-PLNC derived T cell lines

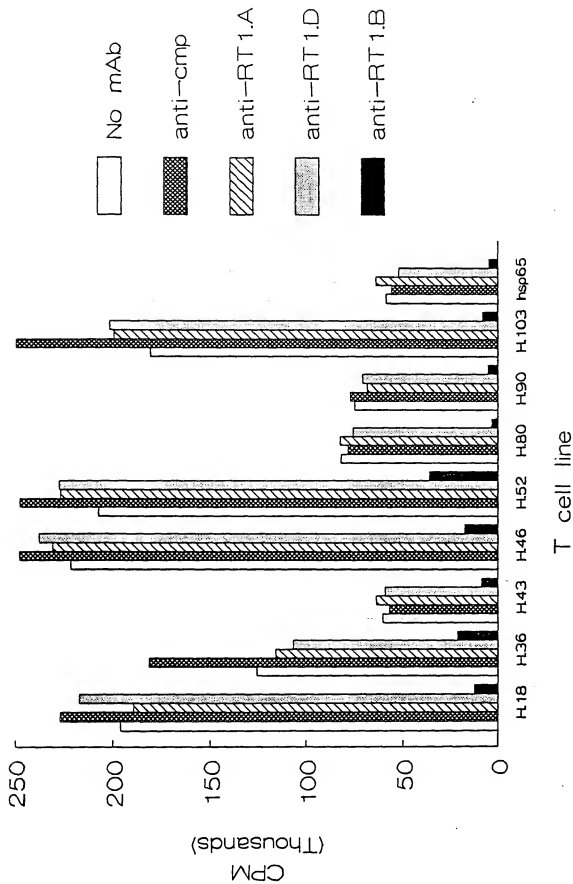☐ hsp65 restim   ■ Mt restim
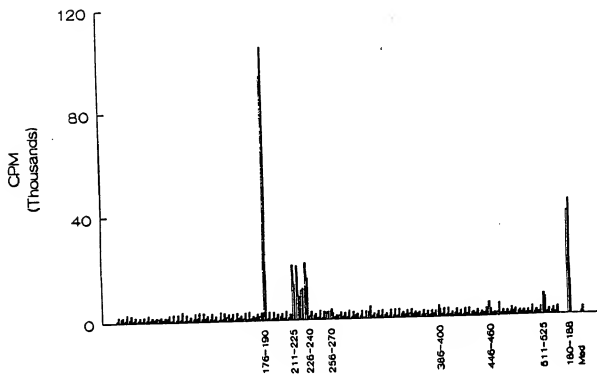
Figure 5

Figure 6

Figure 7

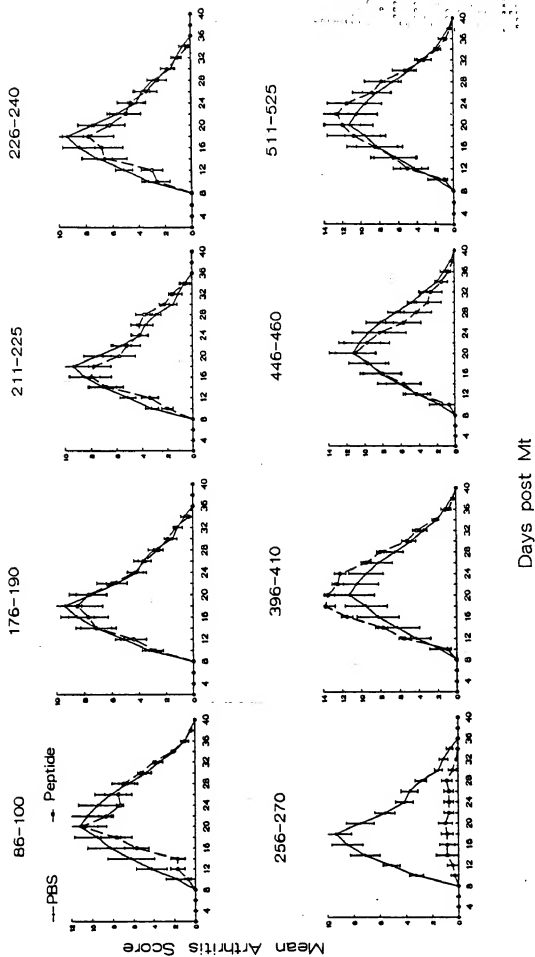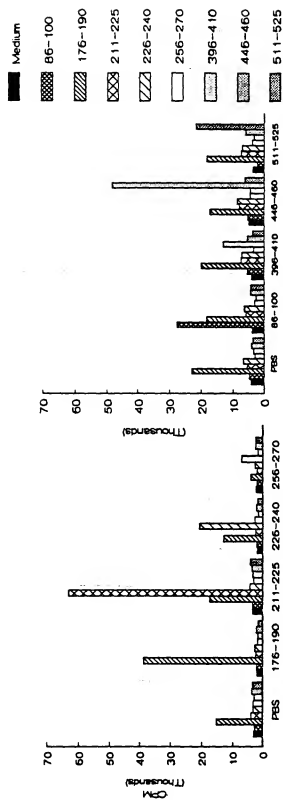Modulation of AA by preimmunization with hsp65 peptides

Figure 8
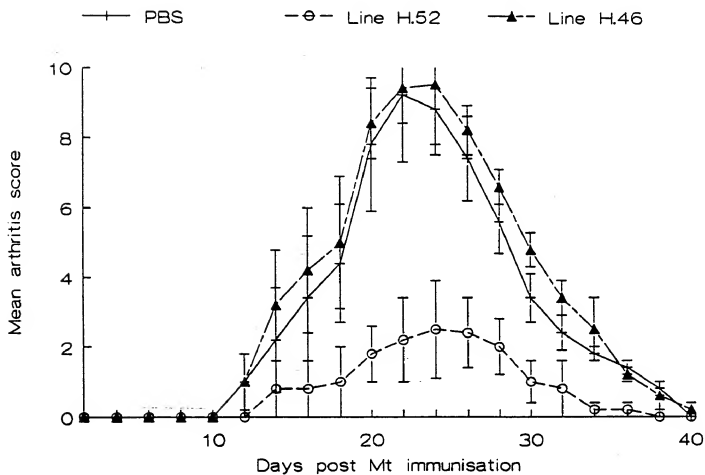
PLNC responses of peptide preimmunized rats

Figure 9

Modulation of AA by T cell lines

19月

Figure 10

Modulation of CP20961-induced arthritis
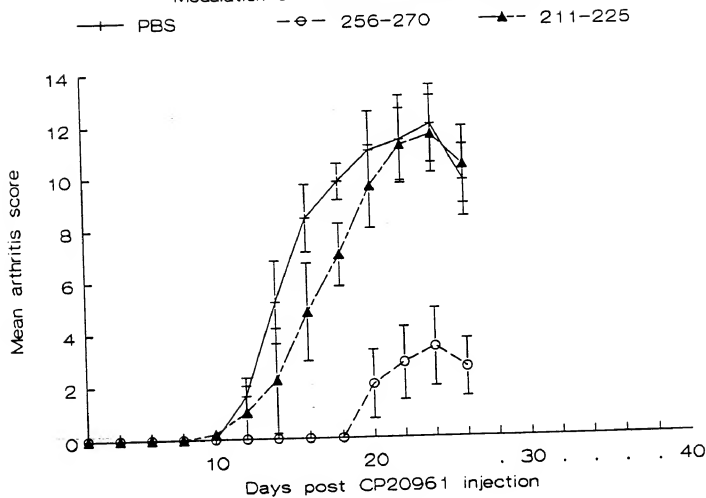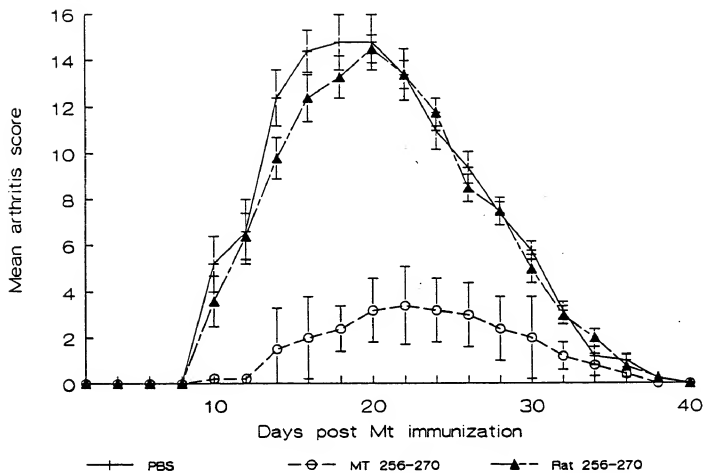
PBS ─┼─   256-270 ─⊖─   211-225 ─▲──

Figure 11

Preimmunization with rat hsp60(256-270) fails to protect against AA

Figure 12

Responses of T cell lines from rats immunized with mycobacterial or rat 256-270

- APC
+APC
Mt
251-265
256-270
261-276
256-266
Rat
251-265
256-270
261-270
256-265

0   20   40   60   80   100

CPM   (Thousands)

P.m52   P.r57